Removal of triazine herbicides from freshwater systems using photosynthetic microorganisms

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Abstract

The uptake of the triazine herbicides, atrazine and terbutryn, was determined for two freshwater photosynthetic microorganisms, the green microalga *Chlorella vulgaris* and the cyanobacterium *Synechococcus elongatus*. An extremely rapid uptake of both pesticides was recorded, although uptake rate was lower for the cyanobacterium, mainly for atrazine. Other parameters related to the herbicide bioconcentration capacity of these microorganisms were also studied. Growth rate, biomass, and cell viability in cultures containing herbicide were clearly affected by herbicide uptake. Herbicide toxicity and microalgae sensitivity were used to determine the effectiveness of the bioconcentration process and the stability of herbicide removal. *C. vulgaris* showed higher bioconcentration capability for these two triazine herbicides than *S. elongatus*, especially with regard to terbutryn. This study supports the usefulness of such microorganisms, as a bioremediation technique in freshwater systems polluted with triazine herbicides.

Keywords

Bioaccumulation; Terbutryn; Atrazine; Microalga; Cyanobacterium

1. Introduction

Triazines are widely used herbicides that are highly toxic and frequently appear in natural watercourses, atrazine being one of the most frequently detected pesticides in aquatic systems (Graymore et al., 2001 and Rebich et al., 2004). Atrazine (6-chloro-*N*²-ethyl-*N*⁴-isopropyl-1,3,5-triazine-2,4-diamine) and terbutryn (*N*²-*tert*-butyl-*N*⁴-ethyl-6-methyl-thio-1,3,5-triazine-2,4-diamine) are pre- or post-emergence systemic herbicides. They are used to control most grasses and many annual broad-leaved weeds in winter cereals, potatoes, legumes,

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sunflowers, maize, sugar cane and citrus fruit. Furthermore, terbutryn is used as an aquatic herbicide for controlling submerged and free-floating weeds and algae in watercourses (Tomlin, 2003). Different studies have demonstrated that freshwater organisms, particularly freshwater microalgae, are most affected by these pollutants, more than marine organisms (Solomon, 1996).

These herbicides affect the main energy production process in green cells, the photosynthesis (Védrine et al., 2003). Some studies have indicated that different algal species have different sensitivity to triazine herbicides, and responses vary widely depending on the species tested and the concentrations used (Blank et al., 1984 and Ma and Chen, 2005). Differences in the uptake of this type of herbicides by microalgal cells result from a complex interaction between several metabolic factors (Kent and Currie, 1995 and Weiner et al., 2004).

Since, the bioconcentration of pesticides, determined by the use of organisms of different trophic levels and taxonomic groups, is an important criteria for ecological hazard assessment for aquatic environments (Manthey et al., 1993 and Singh et al., 1997); the aim of this study was to test the capability of two freshwater microorganisms, the cyanobacterium *S. elongatus* and the chlorophyte *C. vulgaris*, commonly used in toxicity tests, to remove atrazine and terbutryn from culture media.

The results obtained in this work could contribute to test the utility and effectiveness of these microalgae as a bioremediation technique for these herbicides, which could affect higher organisms on longer time scales (McCormik and Cairns, 1994).

2. Materials and methods

2.1. Microalgal cultures

Synechococcus elongatus (*Cyanophyceae*) (strain CCAP 1479/1A) and *Chlorella vulgaris* (*Oocystaceae*) (strain CCAP 211/11B) obtained from the Culture Collection of Algae and Protozoa (CCAP; Cumbria, UK) were grown on sterile Bristol medium (Brown et al., 1967), which was supplemented with different atrazine and terbutryn concentrations (0.025 μM, 0.1 μ M, 0.75 μM). Atrazine and terbutryn were Riedel de Haën Pestanal (RdH Laborchemikalien GmbH & Co.; Seelze, Germany) standards for environmental analysis. Herbicide stock solutions (500 μM) were prepared by dissolving herbicide in 100% methanol and filtered through 0.2 μm filter membranes. Final methanol concentration never exceeded 0.05% (v/v), and no measurable effects in the parameters assayed were observed.

All cultures were carried out in Pyrex glass bottles containing 1500 ml of medium. Bottles were previously rinsed with pure methanol and then with distilled water. Cultures without herbicide were included as positive control, and cultures without microalgae and with herbicide were included as negative control. Initial density of the cultures were 5×10^6 cells ml^{-1} and 9×10^6 cells ml^{-1} for *S. elongatus* and *C. vulgaris*, respectively. For the assays, cells from a 3-day-old culture, in the logarithmic phase of growth, were used. Cultures were illuminated with

fluorescent light (70 ± 2 μmol photon m^{-2} s^{-1}, photosynthetically active radiation), bubbled with air at a rate of 10 l min^{-1}, and maintained at 18 ± 1 °C, with a dark:light cycle of 12:12 h. All experiments were carried out in triplicate.

2.2. Growth measurement

Growth of microalgal cultures under different conditions was measured by counting culture aliquots in a Coulter Epics Flow Cytometer equipped with an argon-ion excitation laser (488 nm), and with detectors of forward and side scatter, and four fluorescence detectors corresponding to four different wavelength intervals: 488–550 nm, 550–600 nm, 600–645 nm, and >645 nm. To exclude non-algal particles, fluorescence of chlorophyll *a* were used, and red fluorescence histograms (>645 nm) were used as gate.

Growth rate (day^{-1}) was calculated using the following formula:

$$\mu = [\ln(N_t) - \ln(N_0)]/\ln 2\ (t - t_0)$$

where N_t is the cell density (cells ml^{-1}) at time t (in days) and N_0 is the cell density at time 0.

Growth data were fitted by a non-linear regression with regression wizard software (Sigma-Plot 8.0, SPSS Inc.).

2.3. Dry biomass

Biomass dry weight was determined according to Utting (1985). Culture aliquots (10 ml) were filtered through previously dried and weighed Whatman GF/C filters. Thereafter, the filters were dried in an oven at 80 °C for 72 h. Values shown are the mean of three replicates.

2.4. Cell viability

Fluorescence of cells stained with propidium iodide (PI; Sigma Chemical Co.) was measured to study cell viability. Propidium iodide is unable to pass through intact cell membranes; however when a cell dies the integrity of the cell membrane fails, and PI is able to enter and stain nucleic acids, producing red fluorescence when excited with blue light(Cid et al., 1996). Red fluorescence was recorded between 600 and 645 nm. Hence, PI can be used to discriminate between live non-fluorescent cells and non-viable fluorescent cells. Aliquots containing 0.5 × 10^6 cells ml^{-1} for the cyanobacterium and 1 × 10^6 cells ml^{-1} for the chlorophyte were treated with PI to a final concentration of 3 μg ml^{-1} for 15 min.

2.5. Determination of herbicide concentrations in cultures

Herbicide concentration in cultures was determined in supernatants resulting from centrifugation of 200 ml aliquots at 3000 × *g* for 15 min at 4 °C. These supernatants were filtered through two superimposed 0.2 μm MF-Millipore filters.

Atrazine and terbutryn were extracted by liquid-solid extraction and further determined by gas chromatography-mass spectrometry (GC-MS). Extractions were carried out in C18 cartridges (Waters; Sep-Pak^{R}Plus) prepared by adding 5 ml of acetonitrile and 5 ml Milli-Q water

successively. Each sample (200 ml) was passed through the cartridge at an approximate speed of 10 ml min^{-1}. Herbicides were eluted from the cartridges with 7.5 ml of ethyl acetate and acetonitrile (1:3). Then solutions were evaporated and resuspended in 1 ml of pure methanol.
Chromatographic separation was carried out with a J&W DB-XLB column (60 m × 0.25 mm × 0.25 μm). The separation procedure includes an increase of the temperature from 70 to 125 °C at a rate of 30 °C min^{-1}, and then to 220 °C at the same rate. This temperature was held during 10 min. Helium was used as a carrier gas. PTV injector was run in solvent split mode using this program: initial temperature, 80 °C; split flow, 20 ml min^{-1}; injection time, 0.5 min; splitless time, 2.5 min; transfer rate, 3.3 °C min^{-1} and final temperature, 300 °C held 10 min. Injection volume was 2 μl.
Mass spectrometry was used for detection and quantification of both triazine herbicides. An ion trap mass spectrometer working in 70 eV electron impact and ion selected monitoring (SIM) was used. For terbutryn, 185 and 226 amu ions were recorded between 25 and 31 min of the chromatogram. The ions selected for the analysis of atrazine were 200 and 215 amu that were recorded between 9 and 25 min of the run. Ion source and transfer line temperatures were set at 250 °C and 290 °C, respectively.

Using this analytical method, the recovery rates of atrazine and terbutryn were 96% and 98%, respectively.

2.6. Determination of herbicides in microalgal biomass

A 200 ml aliquot of each culture was centrifuged at 3000 × *g* for 15 min at 4 °C. The pellet was resuspended in 20 ml of fresh Bristol medium and shaken vigorously. The cell suspension was centrifuged again, and the pellet resuspended in 5 ml of pure methanol, which was kept in darkness for 48 h, and then the cells were disrupted by ultrasounds with two pulses of 30 s. Finally, this methanol suspension was centrifuged again with the aim to obtain a clean supernatant, free of cell debris, which will be used for herbicide analysis as described above.

2.7. Data analysis

Since three microalgal cultures were carried out for each herbicide concentration analysed, and all experiments were conducted three times, data were statistically analysed by a one-way analysis of variance (ANOVA) and, when differences observed were significant, means were compared by the multiple range Duncan test. Significant differences at a level of significance of 0.05 ($P < 0.05$) are represented by an asterisks (*). Data are given as means values ± standard errors of the means.

3. Results

3.1. Growth

Addition of atrazine to the culture medium affected the growth of both species, resulted in a significant (ANOVA test, $P < 0.05$) inhibition of growth only at highest concentrations assayed (Table 1 and Table 2). However, the growth of both species was affected at any terbutryn concentration tested, being the *Chlorella vulgaris* growth arrested (Table 1 and Table 2).

Table 1.

Effect of atrazine and terbutryn on growth rate, dry biomass and viability of *S. elongatus*

Synechococcus elongatus

Herbicide concentration(μM)	Atrazine				Terbutryn			
	Concentration(μg l^{-1})	Growth rate(d^{-1})	Biomass(μg ml^{-1})	Viability (%)	Concentration(μg l^{-1})	Growth rate(d^{-1})	Biomass (μg ml^{-1})	Viability (%)
Control	0	3.23 ± 0.13	160 ± 10	99 ± 2.33	0	3.17 ± 0.08	130 ± 10	99 ± 4.65
0.025	5.39	3.36 ± 0.14	120 ± 10*	98 ± 3.15	6.03	1.26 ± 0.11*	30 ± 10*	95 ± 3.17
0.1	21.57	2.68 ± 0.12*	70 ± 00*	92 ± 1.51	24.40	0.78 ± 0.03*	20 ± 00*	84 ± 2.34*
0.75	161.77	1.11 ± 0.07*	30 ± 00*	90 ± 3.48	181.02	0.14 ± 0.004*	20 ± 00*	80 ± 3.12*

Herbicides were added to the culture medium at the concentrations indicated. Values shown correspond to determinations performed 24 h after herbicide addition.

Table 2.

Effect of atrazine and terbutryn on growth rate, dry biomass and viability of *C. vulgaris*

Chlorella vulgaris

Herbicide concentration(μM)	Atrazine				Terbutryn			
	Concentration(μg l^{-1})	Growth rate(d^{-1})	Biomass(μg ml^{-1})	Viability (%)	Concentration(μg l^{-1})	Growth rate(d^{-1})	Biomass(μg ml^{-1})	Viability(%)
Control	0	0.38 ± 0.01	130 ± 10	99 ± 4.22	0	0.43 ± 0.02	120 ± 00	99 ± 4.32
0.025	5.39	0.45 ± 0.03	120 ± 10*	99 ± 2.31	6.03	0.00 ± 0.02*	70 ± 00*	99 ± 2.56
0.1	21.57	0.39 ± 0.02	120 ± 10*	98 ± 3.54	24.40	0.00 ± 0.00*	70 ± 00*	95 ± 2.35
0.75	161.77	0.00 ± 0.02*	110 ± 10*	95 ± 3.81	181.02	0.00 ± 0.02*	60 ± 10*	94 ± 5.20

Herbicides were added to the culture medium at the concentrations indicated. Values shown correspond to determinations performed 24 h after herbicide addition.

3.2. Dry biomass

In *S. elongatus* cultures, dry biomass (expressed as μg ml^{-1}) decreased significantly ($P < 0.05$) by the addition of herbicides at all concentrations assayed (Table 1). This effect was more important for terbutryn, since the lowest concentration assayed (0.025 μM) reduced four times the dry weight observed in control cultures (Table 1).

In *C. vulgaris* the decrease in biomass of cultures containing either herbicide was also significant for all concentrations assayed (Table 2). In these microalgal cultures, terbutryn reduced twice the dry biomass of control cultures in all concentrations assayed (Table 2).

3.3. Cell viability

Cell viability in *S. elongatus* cultures was affected by the addition of terbutryn(Table 1). In cultures with atrazine concentrations of 0.1 and 0.75 μM, viability was reduced to 92% and 90%, respectively, not differing significantly ($P < 0.05$) from the viability of control cultures (99%). Viability percentage in cultures with concentrations of 0.1 and 0.75 μM terbutryn decreased significantly to 84% and 80%, respectively (Table 1).

In *C. vulgaris* there were no significant differences in viability between control cultures (99%) and cultures with either herbicide, even at the highest concentrations of atrazine (95%) and terbutryn (94%) (Table 2).

3.4. Uptake of herbicides from the culture medium and bioaccumulation in the biomass

3.4.1. Synechococcus elongatus

The percentage of atrazine taken up increased slightly from 10 min to 6 h of exposure, for all concentrations assayed. The maximum difference in the uptake rate was observed between 6 and 12 h (Fig. 1). The percentage of atrazine uptake did not change after twelve hours of culture; in cultures of 0.025 μM uptake was near 80% of the total amount of pesticide in the culture at 12 h, and close to 70% in 0.75 μM cultures (Fig. 1A).

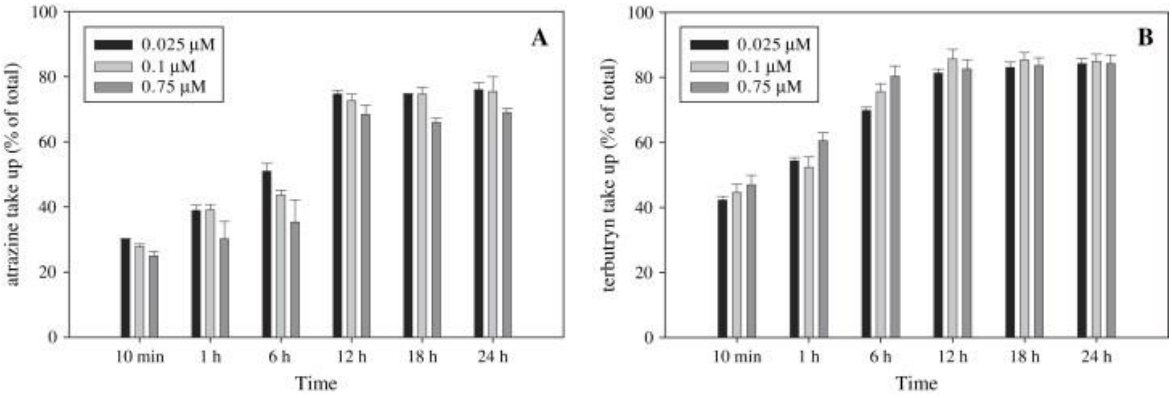

Fig. 1.

Uptake of atrazine and terbutryn by *S. elongatus*. At time zero, the culture medium was supplemented with atrazine (A) or terbutryn (B) at the final concentrations indicated.

In the case of terbutryn the evolution of the uptake was similar to the described above for atrazine, but at the initial time (10 min) the uptake was near 50% including to the highest concentration assayed (Fig. 1B). No differences were observed after 12 h of exposure, removing more than 80% of added terbutryn (Fig. 1B).

Data on herbicide bioaccumulation in microalgal biomass revealed that the limit value of accumulated herbicide is 9 μmol atrazine g^{-1} dry biomass(Fig. 2A), obtained after 12 h of culture, and 12 μmol terbutryn g^{-1} dry biomass (Fig. 2B), at 18 h of culture.

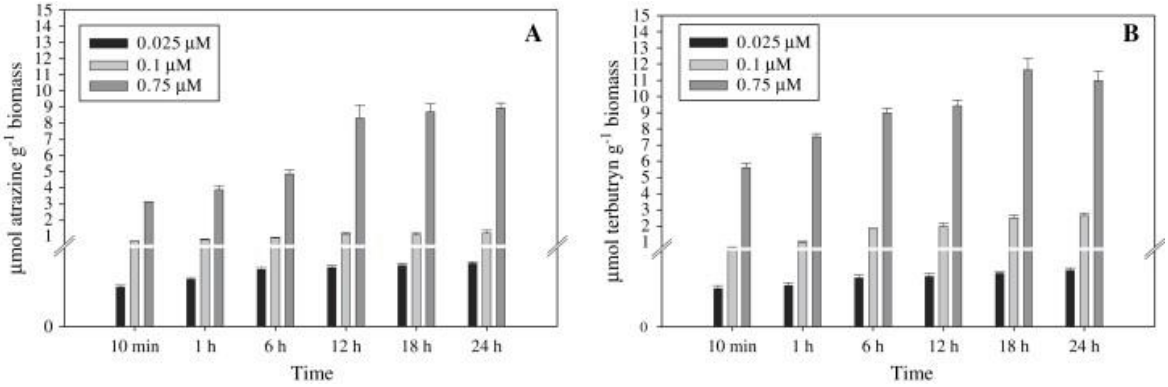

Fig. 2.

Bioaccumulation of triazine herbicides in *S. elongatus* biomass. At time zero, the culture medium was supplemented with atrazine (A) or terbutryn (B) at the final concentrations indicated.

3.4.2. Chlorella vulgaris

The maximum percentage of uptake was after 12 h for either herbicide cultures. Atrazine uptake, at this time, was between 83% (0.75 μM) and 90% (0.1 μM) (Fig. 2A). Terbutryn uptake was between 85% (0.025 μM) and 93% (0.75 μM). After 12 h, the concentrations of both herbicides in all cultures did not vary (Fig. 2B).

Bioaccumulation of herbicides in microalgal biomass had a limit value of 11 μmol atrazine g^{-1} dry biomass (Fig. 4A); this quantity was obtained at six hours of culture, and decreased significantly after 12 h. In the case of terbutryn 12 μmol g^{-1} dry biomass was the limit value of bioaccumulation in *C. vulgaris* biomass, obtained at 18 h of culture (Fig. 4B).

4. Discussion

Triazine herbicides provoke serious damage in natural microalgal populations (Dorigo et al., 2004). Some authors suggest that the bioconcentration of atrazine might be a prerequisite for toxicity in microalgal cells (Tang et al., 1998). In this study some effects provoked by the herbicides are shown. Table 1 and Table 2both show the serious effects on growth for microalgae by herbicide addition to culture medium. The effects on growth of terbutryn seem to be more acute than those of atrazine for either species. *C. vulgaris* cultures growth ceased completely in presence of all terbutryn concentrations assayed (Table 2), suggesting that green microalga are more sensitive to its effects than *S. elongatus*, since the growth of cyanobacterial cultures decreased significantly, but did not cease (Table 1).

But there are other important parameters besides growth in the removal of herbicides from culture medium, as it could be the viability of cells, and the stability of the microalgal biomass after herbicide uptake. The main characteristic of cell death or decrease of cell viability, whether from senescence, acute stress, or aging, seems to be the loss of the ability of cells to maintain

homeostasis (Gahan, 1984). Cellular membranes are selective, dynamic barriers that play an essential role in regulating biochemical and physiological events. The decrease in cell viability, assayed by flow cytometry, is related to the loss of membrane integrity, similar to that which occurs in the aging and senescence process (Thompson, 1988 and Cid et al., 1996). Addition of concentrations of terbutryn higher than 0.1 μM significantly reduces cell viability in *S. elongatus* cultures. This reduction of the cell viability was not observed for any of the remaining cultures of either species (Table 1 and Table 2).

The biomass of the two microalgal species is also affected by the addition of either herbicides (Table 1 and Table 2), mainly by terbutryn which reduced nearly 10% of the biomass of *C. vulgaris* cultures exposed to the highest concentration assayed. Cultures exposed to the concentrations that affected microalgal growth showed cells larger than normal cells, due to the impossibility of completing their cell division. This effect was reported previously in *S. elongatus* cultured with atrazine (González-Barreiro et al., 2004).

The combined effect over growth, stability of microalgal biomass and cell viability must be taken into account when the triazine herbicide bioconcentration capability is studied for the potential application of these microorganisms to environmental problems. Other studies performed with chlorophytes and bacillariophytes revealed that other factors such as cell size and density could provoke differences in atrazine bioconcentration (Weiner et al., 2004).

In *C. vulgaris* cultures exposed to the highest concentration assayed, more than 75% of atrazine was removed from the culture medium after 12 h of exposure (Fig. 3), but posterior analysis showed that herbicide concentration per gram of dry biomass decreased (Fig. 4), while differences in the viability of the microalgal population with respect to the control were not observed. These results indicate that the atrazine bioacumulation expressed as a function of dry biomass could lead to an incorrect interpretation of data, since this value could indicate a release of the herbicide from cells (Fig. 4), that does not occur taking into account the other results (Fig. 3).

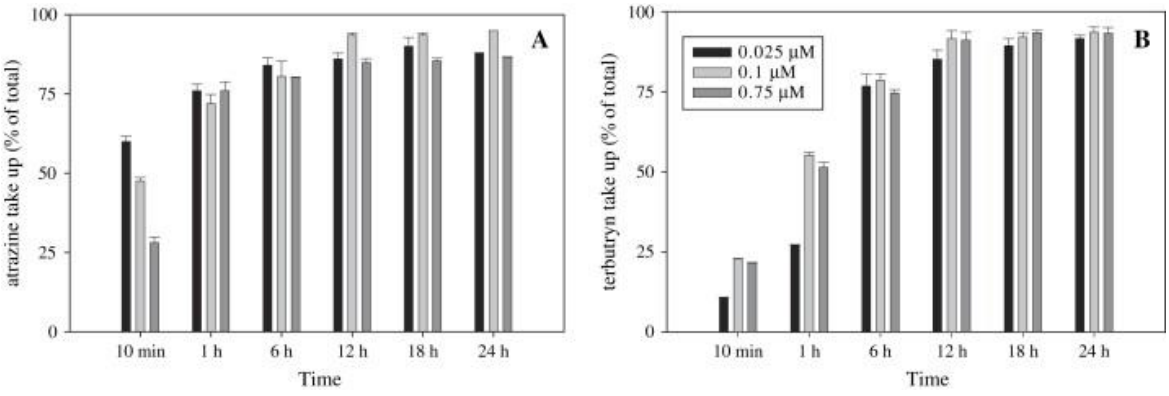

Fig. 3.

Uptake of atrazine and terbutryn by *C. vulgaris*. At time zero, the culture medium was supplemented with atrazine (A) or terbutryn (B) at the final concentrations indicated.

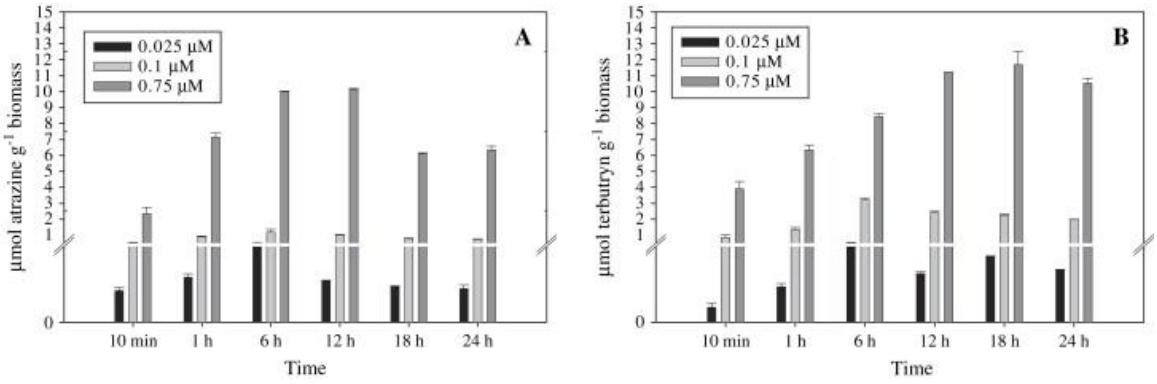

Fig. 4.

Bioaccumulation of triazine herbicides in *C. vulgaris* biomass. At time zero, the culture medium was supplemented with atrazine (A) or terbutryn (B) at the final concentrations indicated.

As indicated previously both herbicides affect the growth of *C. vulgaris* but not cell viability (Table 2). This fact can be translated into a quick bioaccumulation of herbicides in the microalgal biomass. However, in the cyanobacterium cultures exposed to terbutryn, the viability of population decreased significantly, but pesticides are retained in the microalgal biomass, and do not appear in the medium again (Fig. 1 and Fig. 2), even in those cells that are not viable, the pesticide is not liberated. Based on this, it seems that *C. vulgaris*cells are capable of maintaining their cellular integrity whereas *S. elongatus* are not. This fact could be relevant in the effectiveness of uptake of pesticides.

Differences in bioconcentration are also due to the morphological and physiological characteristics of the cells, such as the lipid content of cell membranes and composition of cell walls(Tang et al., 1998). *Chlorella vulgaris* is an eukaryotic microalga whereas *S. elongatus* is a prokaryotic cyanobacteria and their cell walls and membranes are quite different in their lipid composition. Other works reveal the difference in the speed of entrance of different herbicides, such as isoproturon and terbutryn, into *C. vulgaris* as a function of their polarity and their capacity of dilution into the lipids of cell membranes (Rioboo et al., 2002). As shown in this work, speed of entrance of herbicides depends on either the microalgae species and the herbicide assayed (Fig. 1 and Fig. 3). Some authors affirm that the first step in the uptake of herbicides in the sorption of organic compounds is mainly a passive process involving a chemical partitioning into the hydrophobic biomass (Amy et al., 1998). Based on the partitioning coefficient of atrazine (log K_{ow} 2.56) and terbutryn (log K_{ow} 3.49), this herbicide is more lipophilic than atrazine. This can explain the results obtained in which terbutryn is rapidly taken up, as a passive uptake, from the medium by the cells.

5. Conclusions

The results included in this work establish the potential of the microalga *C. vulgaris* and the cyanobacterium*S. elongatus*, for their use in the treatment of waters polluted with triazine herbicides. Actually, *C. vulgaris*seems to be slightly superior to *S. elongatus*, since this microalga exhibits higher cell viability. Moreover, both photosynthetic organisms are amenable

to mass culture production, which would facilitate the establishment of bioremediation systems based on these microorganisms.

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